The Veliger 26(3):229-232 (January 3, 1984)

THE VELIGER

Technique for Narcotizing and Fixing Veliger Larvae of *Amphibola crenata*

by

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Abstract. A three-stage method for narcotizing veliger larvae is described, using calcium-free seawater followed by isotonic $MgCl_2$ and then a benzocaine-procaine mixture. Completely expanded veligers of *Amphibola* can be prepared for scanning and transmission electron microscopy and light microscopy.

INTRODUCTION

To examine development of veliger larvae of the marine pulmonate *Amphibola crenata* (Gmelin), we wished to fix them with the velar lobes extended in the normal swimming position. Initial tests using gradual additon of alcohol and isotonic (7.5%) $MgCl_2$, and bubbling CO_2 through the seawater resulted in retraction of the velum and closure of the operculum, either during the narcotization or upon addition of fixative. Use of chlorobutanol as devised by Bonar & Hadfield (1974) and subsequently employed by Bickell & Chia (1979) with nudibranch veligers was successful with newly hatched *Amphibola* larvae, but even when used at low temperature it caused older larvae to retract. The following three-stage method was developed and resulted in successful fixation for scanning electron microscopy (SEM), which required a high success rate, for transmission electron microscopy (TEM), and for 1 μm plastic sections for light microscopy (LM).

TECHNIQUE AND RESULTS

Veligers were raised from egg masses as described by Pilkington & Pilkington (1982). They were placed in seawater in a solid watch glass and viewed under a dissecting microscope. Sub-stage illumination was used, and this produced a slight warming that seemed to aid the narcotization process. The following procedure applies to larvae in seawater, and appropriate dilutions must be made if lower concentrations are used.

Stage I: Seawater was gradually replaced by calcium-free seawater (with tonicity maintained by slightly increasing magnesium concentration) (Table 1) over a period of about 10 min, using a Pasteur pipette. The intention was to block synaptic transmission, as calcium-free salines have been shown to do this in other molluscan systems (*e.g.*, Berry & Pentreath, 1979). The treatment allowed the larvae to continue swimming, but appeared to block the normal retraction into the shell that is caused by chemical or tactile stimulation.

Stage II: The calcium-free seawater was gradually replaced by isotonic $MgCl_2$, and the larvae were left in this for approximately 5 min while the final narcotizing solution was prepared. Isotonic $MgCl_2$ is a well known narcotic for marine invertebrates (*e.g.*, Grimstone & Skaer, 1972), but resulted in retraction into the shell if applied before Stage I. The $MgCl_2$ solution slowed the swimming rate of the larvae so that, although the cilia were still beating, the larvae gathered at the bottom of the watch glass.

Stage III: The final narcotizing solution contained benzocaine (ethyl-4-aminobenzoate) and procaine hydrochloride (Table 1). The $MgCl_2$ solution was gradually replaced with this mixture, which was added to a full watch glass to minimize turbulence. The veligers were left in it for approximately 10 min until the cilia stopped beating. Benzocaine is a widely used narcotic for fish (*e.g.*, McErlean & Kennedy, 1968), but if applied without Stages I and II caused retraction.

At any stage in the procedure until the cessation of

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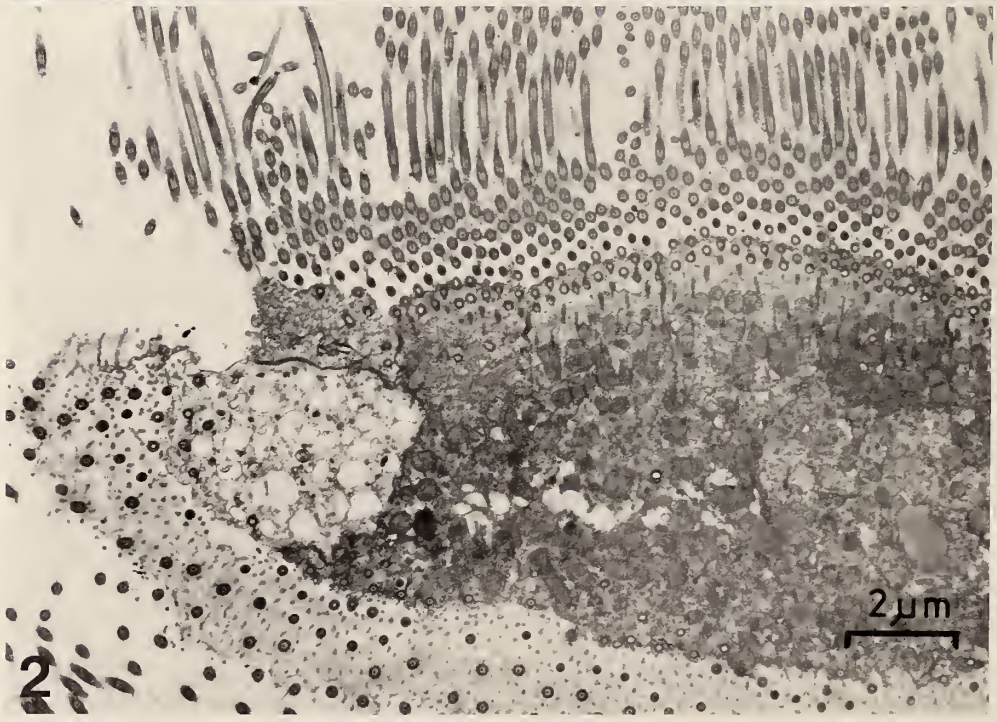

Explanation of Figures 1 and 2

Figure 1. Frontal SEM view of a 4-wk-old veliger with the velar lobes in the extended position. The opening of the mantle cavity can be seen dorsally between the two lobes. Below the velum is the foot and part of the operculum. Scale bar = 50 µm.

Figure 2. TEM view of the velar food groove with pre-oral cilia above and post-oral cilia below. A mass of mucous cells lies internal to the groove. Scale bar = 2 µm.

Table 1

Composition of solutions.

Calcium-free seawater (g/L distilled water)		$MgCl_2$ (g/L distilled water)		Final narcotizing solution (g/5 mL ethanol)		Fixative
NaCl	33.5	$MgCl_2 \cdot 6H_2O$	75	Benzocaine	0.1	2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.6
$MgCl_2 \cdot 6H_2O$	12.3			Procaine	0.002	
Na_2SO_4	3.9			Add to this 45 mL 50% seawater		(Should be made up fresh each day.)
KCl	0.66					
$NaHCO_3$	0.4			(Must be made up immediately before use.)		

ciliary beating, the narcotization can be reversed by replacing the fluid surrounding the larvae with seawater.

Fixation: A few drops of fixative (see Table 1) were added. The fixative reacted with the final narcotizing solution, so most of the fluid in the watch glass was removed rapidly and fresh fixative added. This procedure was repeated twice.

After fixation, veligers were rinsed several times in 0.1 M cacodylate buffer and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer. The larvae were then ready for dehydration and preparation for SEM by critical point drying. For TEM and LM, veligers were embedded in resin. Thick sections (for LM) were cut with glass knives. We did not use decalcification techniques such as those described by Bonar & Hadfield (1974), but cut thin sections (for TEM) using a diamond knife.

Results: Thirty to fifty percent of specimens were fixed successfully with the velum completely extended. The remainder had partly extended velar lobes, and very few were completely closed. The method was more successful with newly hatched veligers than with those 4–5 weeks old and about to metamorphose. Figure 1 shows a 4-wk-old veliger with the velar lobes fully extended. Figure 2 shows a TEM section of one of the velar lobes.

DISCUSSION

Although the early work of Carter (1926) identified nicotine as a useful narcotic for the velar cilia of veliger larvae, Mackie *et al.* (1976) found magnesium chloride more effective as a general narcotic for veligers of *Mangelia.* Our observations showed that $MgCl_2$ did narcotize the veligers of *Amphibola,* but under this narcosis they were still capable of retracting into the shell upon addition of fixative. The procedure described here prevented this reaction.

Although SEM has been a useful tool in describing the shells of larval gastropods (*e.g.,* Robertson, 1972; Thiriot-Quiévreux & Scheltema, 1982), and has been used to examine bivalve larvae (*e.g.,* Lutz *et al.,* 1982), we have been able to find only one previous use of SEM showing veligers with expanded velar lobes (Chia & Koss, 1978). Even in this case, the velum was not completely expanded into the swimming position. We have not yet been able to test the technique described here on other veligers, but if our three-stage approach can be applied to a wide range of gastropod larvae, it should allow wider use of electron microscopy to describe both their external surface and their internal structure.

ACKNOWLEDGMENTS

We are grateful to Gerald Stokes for help with critical point drying and SEM. One of us (C.L.) was supported by a Leverhulme Fellowship and a William Evans Visiting Fellowship while at the University of Otago; P.S. and C.L. wish to thank Professor D. D. McGregor and Professor J. B. Jillett for the facilities provided.

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